

Identification of genes induced by inflammatory cytokines in airway epithelium

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Cooper, Paul, Suzanne Potter, Beatrice Mueck, Shida Yousefi, and Gabor Jari. Identification of genes induced by inflammatory cytokines in airway epithelium. *Am J Physiol Lung Cell Mol Physiol* 280: L841–L852, 2001.—Epithelial cells lining the airways are thought to play a prominent role in respiratory diseases. We utilized cDNA representational difference analysis to identify the genes in which expression is induced by the proinflammatory cytokines tumor necrosis factor- α and interleukin-1 β in primary human bronchial epithelial cells and hence are relevant to airway inflammation. Hybridization of the subtraction product to arrayed cDNAs indicated that known tumor necrosis factor- α and interleukin-1 β -inducible genes such as B94, Zfp36, and regulated on activation normal T cell expressed and secreted were represented, confirming the success of the subtraction experiment. A 1,152-clone library potentially representing genes with higher transcript levels in cytokine-treated human bronchial epithelial cells was generated and sequenced. Sequence similarity searches indicated that these clones represented 57 genes of known function, 1 gene of unknown function, 6 expressed sequence tags, and 2 novel sequences. The expression of 19 of these clones was studied by a combination of Northern blotting and RT-PCR analyses and confirmation of differential expression for 10 known genes, 2 expressed sequence tags, and a novel sequence not represented in any of the public databases was obtained. Thus cDNA representational difference analysis was utilized to isolate known and novel differentially expressed genes, which putatively play a role in airway inflammation.

representational difference analysis; human bronchial epithelial cells; tumor necrosis factor- α ; interleukin-1 β ; airway disease

THE AIRWAY EPITHELIUM has an important role in many of the responses seen in respiratory diseases. It constitutes the interface between the internal milieu and the external environment. It is the first point of contact for inhaled substances, respiratory viruses, and airborne allergens. It is also a primary target for inhaled drugs. The airway epithelial cells are classically considered to provide the physical barrier between the external and internal environments. A recent study (1) has indicated that airway epithelial cells can act as effector cells, responding to a variety of exogenous and/or endogenous stimuli by generating and releasing addi-

tional mediators of inflammation such as cytokines, eicosanoids, and reactive oxygen species, thereby appearing to play an important regulatory role in inflammatory and immune processes. Airway epithelial cells are known to produce as well as respond to the potent inflammatory cytokines interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α). These two cytokines have been implicated in the pathogenesis of many acute and chronic inflammatory as well as autoimmune diseases including septic shock, rheumatoid arthritis, and inflammatory bowel disease. They are also believed to play an important role in the respiratory inflammation observed in chronic bronchitis, chronic obstructive pulmonary disease, and asthma (14, 15, 40, 45).

TNF- α and IL-1 β are produced by a wide variety of immune and structural cells including monocytes, macrophages, T cells, and endothelial and epithelial cells, and they exert many shared biological effects on a number of different target cells. They appear to activate the same set of transcription factors, primarily nuclear factor- κ B (NF- κ B) and activator protein-1, even though they signal through distinct receptors (16). In turn, these two transcription factors govern the expression of many genes that are involved in the immune responses and inflammatory conditions. Genes responding to the effect of TNF- α and IL-1 β via the action of these transcription factors include cytokines, growth factors, cell adhesion molecules, metalloproteinases, and other inflammatory mediators (3, 28).

Airway epithelial cells may participate in the development and maintenance of these respiratory diseases through different mechanisms. They may initiate and help sustain inflammation through the release of inflammatory mediators and by contributing to cell-specific recruitment and activation of granulocytes and other leukocytes through differential expression of proadhesive signals and/or chemotactic factors and cytokines (7). In addition, during the course of sustained inflammation, the airway epithelium may also be involved in the remodeling process that is typical of obstructive lung diseases through the release of growth factors and profibrotic signals (30).

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The identification of genes in which expression is altered in the inflamed airway epithelium can help to delineate the molecular mechanisms of the inflammatory processes observed in respiratory diseases. Our recent studies have focused on categorizing the expression pattern of transcripts and associating the changes to cellular events that ultimately contribute to the determination of the respective function of the identified genes. cDNA representational difference analysis (cDNA-RDA) is a sensitive, PCR-based subtractive enrichment procedure that can be used for the isolation of genes with altered expression between various tissues or cell samples (23, 32). We performed cDNA-RDA to identify the genes differentially expressed between TNF- α - and IL-1 β -stimulated primary human bronchial epithelial cells (HBECs) and unstimulated control cells.

Here we describe the identification of the transcripts more abundantly expressed in HBECs stimulated with TNF- α and IL-1 β . Several of these genes have previously been reported as being more actively transcribed with cytokine stimulation in various cell and tissue types (6, 8, 22, 26, 27, 29, 33–35, 37–39, 43). We also identified two uncharacterized expressed sequence tags (ESTs), one of which showed preferential expression in the adult lung along with a sequence not represented in the public databases that is predominantly expressed in adult and fetal lungs. Identification of the genes central to epithelial airway inflammation may provide novel targets that may serve to modify the inflammatory condition and, ultimately, the progression of respiratory disease.

MATERIALS AND METHODS

Cell culture. Primary HBECs obtained from Clonetics were seeded at a density of 2.5×10^6 cells/ml and grown to 70–80% confluence in the medium supplied (Clonetics). Actively growing cells were simultaneously stimulated with TNF- α (100 ng/ml) and IL-1 β (100 ng/ml) for 4 h (9). Control cells were incubated in medium for the same length of time, and then both cell populations were harvested.

RNA isolation and cDNA synthesis. Total RNA was extracted with TRIzol reagent (GIBCO BRL, Life Technologies) as described by the manufacturer. mRNA was purified with the MessageMaker reagent assembly kit (GIBCO BRL) with conditions recommended by the manufacturer. For cDNA-RDA, 300 ng of mRNA were used to synthesize double-strand cDNA with the Superscript choice system (GIBCO BRL). For RT-PCR analysis, first-strand cDNA was synthesized from 5 μ g of total RNA that had been DNase treated with RNase-free DNase I (Roche Diagnostics).

Generation of subtracted libraries by cDNA-RDA. cDNA-RDA was performed essentially as described by O'Neill and Sinclair (32). Briefly, double-strand cDNA was extracted once with an equal volume of phenol-chloroform-isomyl alcohol and precipitated with 0.5 volume of 7.5 M ammonium acetate and 2.5 volumes of ethanol. Molecular biology-grade chemicals were purchased from Sigma, and the cDNA precipitation conditions used were as recommended by the manufacturer of the cDNA synthesis kit (GIBCO BRL). After centrifugation, the resultant pellet was washed with 70% ethanol and resuspended in 16 μ l of sterile water. Double-strand cDNA

was digested with *DpnII* (New England Biolabs) and ligated to R-adaptors (a 24-mer annealed to a 12-mer). Amplicons for both "tester" (cDNA prepared from stimulated HBECs) and "driver" (cDNA prepared from unstimulated control HBECs) were generated with Expand Long Template PCR system and Expand Buffer 1 (Roche Diagnostics). Typically, five 100- μ l PCR for each tester and 10 PCR for each driver were performed. *DpnII* digestion was used to remove the R-adaptors from both driver and tester amplicons followed by ligation of J-adaptors to the tester. Subtractive hybridizations were performed in 5- μ l reactions at 67°C for 20 h in a GeneAmp 2400 (PerkinElmer). To generate difference product one (DP1), 250 ng of tester cDNA were mixed with 25 μ g of driver cDNA at a ratio of 1:100. DP1 cDNA was digested with *DpnII* to remove J-adaptors before ligation of N-adaptors. To generate difference product two (DP2) from DP1, 31.25 ng of tester were mixed with 25 μ g of driver cDNA at a ratio of 800:1. Digested and excess adaptors were removed by washing the cDNA on Microcon 30 filters (Amicon) as recommended by O'Neill and Sinclair (32).

Cloning of difference products. DP2 was amplified with the primer 5'-CUACUACUACUACUAGGCAACTGTGCTATCCGAGGGAA-3' (N-Bgl-24dU) and cloned into the pAMP10 vector with the ligation-independent cloning protocol provided with the CloneAMP kit (GIBCO BRL). Plasmids were purified from 3-ml cultures of the white colonies with the Wizard Plus Miniprep DNA purification kit (Promega).

DNA sequencing and analysis. Cycle sequencing of plasmid DNA was performed on an automated ABI310 or ABI377 sequencer (PerkinElmer) with M13 forward and reverse primers according to the manufacturer's instructions. Sequence similarity searches were performed with the BLAST algorithm (2), and sequence alignments were generated with the GCG software package (Wisconsin Package version 9.1).

Northern and "virtual" Northern blots. For Northern blot analyses, a master mix of mRNA or total RNA from unstimulated and cytokine-stimulated HBECs was combined with RNA loading buffer (Sigma), and aliquots were loaded for the generation of eight 1- μ g mRNA blots and eight 20- μ g total RNA blots. RNA was electrophoresed in 1% agarose formaldehyde denaturing gels for 2 h. RNA was transferred to Hybond-N membranes (Amersham) by capillary transfer with 20 \times saline-sodium citrate (SSC), and ultraviolet cross-linked to the filter. Virtual Northern blots (= Southern blots of cDNA) were prepared with the same unsubtracted cDNA representations that were used as the tester or driver during the enrichment procedures. Five hundred nanograms of amplicon cDNA were electrophoresed in 1.5% agarose gels and blotted by capillary transfer.

Hybridization analyses. Clone inserts for the hybridization analysis were generated by PCR amplification from plasmid DNA with the N-Bgl-24 primers (32). Probes for DP2 cDNA and amplified plasmid inserts were synthesized by random priming of 25 ng of DNA in the presence of [α - 32 P]dCTP (Amersham) with the Rediprime random-primer labeling system (Amersham). All hybridizations were performed overnight in ExpressHyb hybridization solution (Clontech) at 65°C. After hybridization, the Northern and RNA Master blots (Clontech) were washed in 2 \times SSC-0.05% SDS for 20 min at room temperature followed by two 20-min washes in 0.1 \times SSC-0.1% SDS at 50°C. For Southern blots and GeneFilters cDNA arrays (Research Genetics), three consecutive 15-min washes were performed at 65°C in solutions containing 0.5 \times SSC-0.1% SDS followed by 0.2 \times SSC-0.1% SDS and finally 0.1 \times SSC-0.1% SDS. The filters were exposed to phosphorimager plates for between 2 h and 5 days and were visualized by a STORM 840 phosphorimager

(Molecular Dynamics). Representative HBEC Northern and Southern blots were hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Ambion) for normalization of transcript intensities. Individual blots were stripped and reprobed no more than 3 times to ensure that no significant reduction of signal intensity occurred. Multiple tissue Northern blots were hybridized with a GAPDH probe (Ambion) to control for loading. GeneFilters hybridization results were analyzed with the Pathways software (Research Genetics).

Semiquantitative RT-PCR analysis. Semiquantitative RT-PCR analysis for GAPDH, IL-8, the Epstein-Barr virus (EBV)-induced gene 3 (EBI3), EXER107, and EXER102 was performed. Briefly, 100 ng of single-strand cDNA were used to seed a 50- μ l PCR mixture and were subjected to 22–36 cycles of PCR (95°C for 20 s, 68–60°C for 20 s, and 72°C for 20 s) in a PerkinElmer DNA thermal cycler. After the designated number of cycles, 7 μ l of the reaction mixture were removed before the samples were returned for further cycling. The PCR products were visualized on 1.5% agarose gels stained with 0.5 μ g/ml of ethidium bromide. Scanned gel images were imported into ImageQuant 5.0 (Molecular Dynamics), and the volume density of the amplified products was calculated.

RESULTS

cDNA-RDA using unstimulated and cytokine-stimulated HBEC mRNA. Cultured primary HBECs were used to mimic *in vivo* inflammation and to identify cytokine-responsive genes with potential relevance to inflammatory airway diseases. Initially, we determined the optimal stimulation period for the HBECs cultured with TNF- α and IL-1 β . After stimulation, RNA was harvested after 1, 2, 4, 8, and 24 h of incubation. Semiquantitative RT-PCR relative to a GAPDH control was performed with several genes, including those for IL-8, intercellular adhesion molecule-1 (ICAM-1), IL-6, and TNF- α , known to be important in airway inflammation and induced by TNF- α and IL-1 β (27, 34, 43). Optimal stimulation was determined as being 4 h after the initial addition of the two cytokines (results not shown). cDNA-RDA was then performed with unstimulated and cytokine-stimulated HBEC mRNA samples. To isolate genes more actively transcribed in cytokine-stimulated HBECs, we used cDNA from cytokine-stimulated HBECs as the tester and cDNA from unstimulated HBECs as the driver. Two rounds of enrichment were used to generate DP2 because these conditions have previously been shown to be sufficient for removing unwanted background and to enrich for differentially expressed fragments (32). To determine whether the subtraction experiment had been successful in enriching for sequences expressed at a higher abundance in the tester population, we hybridized the DP2 population to high-density cDNA arrays. Each of the two cDNA filters (GeneFilters GF200 and GF202) contains ~5,000 arrayed sequences. For GF200 and GF202, ~50 and 10%, respectively, of these sequences were highly similar to or represent genes of known function, the remaining sequences on the filters represent ESTs with unknown function. To determine which genes were potentially

represented in DP2, hybridization results were analyzed with the Pathways software (Research Genetics). Table 1 summarizes these results and demonstrates that many of the known genes detected on these filters have previously been shown to be upregulated by TNF- α and/or IL-1 β in a variety of other cell and tissue types.

Sequence analysis of subtraction products. The cDNA species present in DP2 that had been enriched for genes that were upregulated by cytokine stimulation were cloned into the pAMP10 vector and further characterized by DNA sequence analysis. To determine the redundancy of the library, inserts from 72 clones were amplified and digested with the restriction enzymes *Rsa*I and *Alu*I and analyzed on a 1.5% agarose gel. After inspection of the digestion products, 48 clones were chosen for sequencing. Comparison of these sequences with those in the public databases resulted in the identification of 18 known and novel genes (results not shown). To determine the redundancy in the library, 384 clones from this library were arrayed on filters, and probes representing these 18 genes were individually hybridized to the arrays. Results from this analysis indicated that only 88 clones from this 384-clone library (~23% of the library) gave hybridization signals. These data indicated that the DP2 cDNA population potentially contained gene fragments representing many more genes. We therefore generated and sequenced a 1,152-clone cDNA library. Sequence information was collected, vector sequences were removed, and the remaining sequences were assembled into clusters. Sequences representing the individual clusters were compared with those present in the public databases. Sixty-six different sequences were represented by the 1,152 clones. Of these, 57 were identical to known genes, 1 sequence (KIAA0058) identified a protein of unknown function, 6 identified human ESTs (EST1–6), and 2 sequences did not show any similarity to any known sequences (EXER107 and UNK2). Table 2 summarizes the results of this analysis. Twelve clones contained the sequence from the R-Bgl-24 adaptor, the sequence used in the generation of the original representations. These clones most probably arose from an inefficient digestion with the *Dpn*II restriction enzyme, and hence the R-adaptors were not totally removed, resulting in cDNA fragments that would behave effectively as tester unique fragments in the subtractive hybridization step. Clones identified as containing the R-adaptor sequence were not analyzed further.

Expression analysis of individual transcripts. Because methods based on subtractive enrichment procedures have been shown to yield false positives, the expression pattern of individual clones was further verified by virtual Northern and Northern blotting and semiquantitative RT-PCR analysis. For virtual Northern and Northern analyses, cDNA inserts were isolated from the sequenced clones and used as hybridization probes on blots containing unstimulated and stimulated cDNA and mRNA (Figs. 1 and 2). Not all se-

Table 1. Summary of genes identified by hybridization of DP2 to GeneFilters GF200 and GF202 cDNA arrays

Intensity	Field	Grid	Column	Row	Cluster ID	cDNA ID	GenBank Accession No.
<i>Proteases and antiproteases</i>							
225.6	1	F	3	2	Hs.75557	22040	T72581
93.1	2	H	11	9	Hs.22239	30850	R42500
230.4	1	G	7	10	Hs.79572	264117	N20475
264.8	1	F	11	26	Hs.84898	898035	AA598950
263.7	1	E	2	11	Hs.1417	240766	H80215
243.8	1	G	2	22	Hs.75621	123561	R00822
258.3	2	G	4	2	Hs.14804	109316	T80924
<i>Adhesion molecules</i>							
235.7	1	F	12	5	Hs.79133	40751	R56219
266.0	1	F	12	6	Hs.78206	69672	T53626
248.5	1	G	6	7	Hs.82004	251019	H97778
224.7	1	F	11	27	Hs.58464	774754	AA442092
335.6	1	A	2	26	Hs.82848	149910	H00766
315.9	1	A	2	27	Hs.83646	186132	H39660
236.6	1	A	3	24	Hs.832	340644	W55709
245.3	1	H	8	29	Hs.851	773332	AA425451
253.3	1	G	2	29	Hs.78146	130541	R22412
227.5	1	D	10	11	Hs.20313	180298	R85257
<i>Transcription factor</i>							
489.1	1	A	7	25	Hs.1665	23804	R38383
221.8	1	F	6	19	Hs.765	126368	R06446
<i>Cytokines</i>							
167.0	1	D	2	7	Hs.126256	324655	W47101
229.6	1	A	2	22	Hs.340	768561	AA425102
430.4	1	A	2	16	Hs.69744	840753	AA486072
<i>Membrane proteins</i>							
231.8	1	A	3	26	Hs.46459	755054	AA482637
462.7	1	E	3	12	Hs.23582	809938	AA454810
225.7	1	A	7	24	Hs.82685	813552	AA455448
274.5	1	H	12	2	Hs.25856	739183	AA421296
260.8	1	B	12	3	Hs.46437	842860	AA486393
129.0	1	A	4	26	Hs.106671	41070	R56100
<i>Apoptosis</i>							
238.5	1	D	10	24	Hs.38768	814478	AA459263
<i>Growth factors</i>							
223.8	1	B	2	9	Hs.73793	34778	R19956
<i>Unassigned</i>							
5757.2	1	D	7	14	Hs.75522	810444	AA457114
286.1	1	G	3	15	Hs.25590	547247	AA085318

Continued

quences were tested for differential expression because many genes have been previously reported as having induced expression with TNF- α and IL-1 β . We tested 31 sequences by virtual Northern analysis for the dif-

ferential abundance between tester and driver cDNAs. We detected an increased hybridization intensity in cDNA generated from cytokine-stimulated HBECs for 23 of these sequences (Fig. 1), 3 of the sequences did

Table 1.—Continued

Title	Upregulation Reported, Ref. No.	Gene	Chromosome	STS Name
<i>Proteases and antiproteases</i>				
Matrix metalloproteinase-2 (gelatinase A; collagenase type IV)	6*†	MMP2	16q13	WI-7659
<i>Homo sapiens</i> mRNA for putative MT4-MMP protein (GF202)				
Cathepsin D (lysosomal aspartyl protease)		CTSD	11p15.5	
Cathepsin B‡	26*	CTSB	8p22	SHGC-9711
Tissue inhibitor of metalloproteinase-1 (erythroid potentiating activity, collagenase inhibitor)	6*†	TIMP1		
Protease inhibitor 1 (antielastase), α_1 -antitrypsin		PI	14q32.1	SHGC-32639
ESTs, highly similar to α_1 -antichymotrypsin precursor (<i>H. sapiens</i>)‡	33*			
<i>Adhesion molecules</i>				
Cadherin 5		CDH5		SHGC-15929
Cadherin 5, VE-cadherin (vascular epithelium)		CDH5	16q22.1	SHGC-13798
<i>H. sapiens</i> mRNA for E-cadherin				SHGC-11833
Catenin (cadherin-associated protein), β 1 (88 kDa)		CTNBN1	3p22-p21.3	
L-selectin (lymphocyte adhesion molecule-1)		SELL	1q23-q25	WI-7045
E-selectin (endothelial adhesion molecule-1)	38*†	SELE	1q23-q25	WI-9125
Integrin, β 8		ITGB8	7	sWSS2601
Integrin, α E (antigen CD103, human mucosal lymphocyte antigen-1; α -polypeptide)		ITGAE	17	
Platelet endothelial cell adhesion molecule (CD31 antigen)		PECAM1	17q23	WI-7753
Human cell adhesion kinase- β (CAK β) mRNA, complete cDNA sequence (cds)			8	WI-12748
<i>Transcription factor</i>				
Zinc finger protein homologous to Zfp-36 in mouse	8*	ZFP36	19q13.1	
GATA-binding protein-1 (globin transcription factor-1)		GATA1	Xp11.23	
<i>Cytokines</i>				
Interleukin-1 β (GF202)*‡		IL1B	2q13-q21	CHLC.UTR_00699_X04500
Small inducible cytokine A2 (monocyte chemoattractant protein-1, homologous to mouse Sig-je)	22*	SCYA2	17q11.2-q12	WI-7605
Small inducible cytokine A5 (RANTES) (GF202)	35*†	SCYA5	17q11.2-q12	CHLC.UTR_03943_M21121
<i>Membrane proteins</i>				
Human putative transmembrane receptor IL-1 receptor-related protein mRNA, complete cds [interleukin-1 β receptor 1 (IL-18R1)]				
Membrane component, chromosome 1, surface marker 1 (40-kDa glycoprotein, identified by monoclonal antibody GA733)		M151	1p32-q12	WI-7569
CD47 antigen (Rh-related antigen, integrin-associated signal transducer)		CD47	3q13.1-q13.2	WI-8002
CD68 antigen		CD68		
Cytokine receptor family II, member 4		CRFB4	21q22.1	CHLC.UTR_04215_Z17227
ESTs, highly similar to thymic epithelial cell surface antigen (<i>Mus musculus</i>) (GF202)				
<i>Apoptosis</i>				
Human Bcl-2 related (Bfl-1) mRNA, complete cds‡	29*†		15	SHGC-35552
<i>Growth factors</i>				
Vascular endothelial growth factor	37*	VEGF	6p12	SHGC-9780
<i>Unassigned</i>				
B94 protein†	39*		14	
Human stanniocalcin precursor (STC) mRNA, complete cds‡			8	STS_U25997

DP2, difference product 2; MT4-MMP, membrane type 4 matrix metalloproteinase; EST, expressed sequence tag; RANTES, regulated on activation normal T cell expressed and secreted. References are for previous studies that reported upregulation of the transcript or protein in airway epithelial cells or other cell systems by tumor necrosis factor- α (*) or interleukin (IL)-1 β (†). ‡Fragments from these genes were also identified in the sequenced library cloned from DP2.

not show appreciably different hybridization intensities between the 2 lanes, and 5 sequences did not generate a hybridization signal (Table 2). Nineteen sequences were analyzed by Northern blotting, and

differential expression was shown for 10 known genes, 2 ESTs (EXER102 and EST3), and a novel sequence (EXER107; Fig. 2) compared with a GAPDH control. Six sequences did not show any obvious visible expres-

Table 2. Summary of genes identified by sequencing of DP2 library

Gene Product	Differential Expression		Gene Product	Differential Expression	
	Virtual Northern	Northern		Virtual Northern	Northern
Membrane proteins			Housekeeping		
ICAM-1	Yes		α -Enolase		
<i>H. sapiens</i> syndecan 4 (amphiglycan, ryudocan) (SDC4)	Yes	Yes	<i>H. sapiens</i> β -actin (ACTB)		
Human cytokine receptor (EBI3)*	ND	Yes	Human γ -actin		
Annexin VIII (ANX8)	Yes		β -Tubulin		
Cytokines/chemokines/secreted proteins			β -Tubulin-like		
ENA-78	Yes	Yes	Lactate dehydrogenase-A (LDH-A)		
Human follistatin	Yes	Yes	<i>H. sapiens</i> aldehyde dehydrogenase 6 (ALDH6)		
IL-1 β †	Yes		Human acidic ribosomal phosphoprotein P0 mRNA		
Tumor necrosis factor (TNF- α)			<i>H. sapiens</i> mitochondrial DNA		
Granulocyte-macrophage colony-stimulating factor (GM-CSF)			Keratins		
Granulocyte colony-stimulating factor (G-CSF)			Human type II keratin K5		
Proteases/antiproteases			<i>H. sapiens</i> keratin 6		
MMP1	Yes		Human mesothelial keratin K7		
MMP3		Yes	Keratin K19		
MMP9			ESTs		
MMP10			EXER102	ND	Yes
Urokinase-type plasminogen activator	Yes	Yes	EST3	Yes	Yes
Tissue-type plasminogen activator (tPA)	Yes	NS	EST4	Yes	
<i>H. sapiens</i> mRNA for trypsinogen IV a-form	NS		EST5	ND	
<i>H. sapiens</i> α_1 -antitrypsin†	Yes	NS	Protein of unknown function		
Cathepsin B†			Human mRNA for KIAA0058	Yes	NS
Apoptosis			Novel		
Human Bel-2 related (Bcl-1), BCL2A1, GRS†	Yes	Yes	EXER107	Yes	Yes
Human lamin B2 (LAMB2)	Yes	NS	Clones containing R-Bgl-24 sequence		
Kinases			Human membrane glycoprotein 4F2		
<i>H. sapiens</i> protein kinase, mitogen-activated kinase 3	Yes	Yes	<i>H. sapiens</i> cellular retinoic acid-binding protein 2 (CRABP2)		
Human mRNA for phosphoinositide kinase II β	Yes	NS	Transcriptional intermediary factor-1 β		
Unassigned			zinc finger protein, nuclear corepressor KAP-1		
B94†	Yes	Yes	<i>H. sapiens</i> rabkinesin6		
Stanniocalcin (STC1)†	Yes		<i>H. sapiens</i> Kunitz-type protease inhibitor (kop)		
<i>H. sapiens</i> B-factor, properdin (BF), complement B	Yes	NS	<i>H. sapiens</i> CGI-69 protein		
<i>H. sapiens</i> X (inactive)-specific transcript (XIST)	ND		Human 1-5U gene from interferon-inducible gene		
Human thyroid hormone binding protein (p55)	ND		<i>H. sapiens</i> fibulin 1 (FBLN1)		
Platelet-type phosphofructokinase			<i>H. sapiens</i> phosphatidylinositol synthase (PIS)		
Human transglutaminase mRNA					
Stress/antioxidant proteins					
Human 90-kDa heat shock protein	Yes	Yes	EST1		
Mn superoxide dismutase	Yes		EST6		
Human glutathione S-transferase (P1b)			UNK2		
Signal transduction					
Human epithelial cell marker protein 1 (HME1) 14-3-3 α -protein	NS				
<i>H. sapiens</i> moesin (MSN)	NS				

ICAM-1, intercellular adhesion molecule-1; ENA-78, epithelial cell-derived neutrophil-activating protein-78; ND, not detected; NS, not shown. *Genes tested by RT-PCR. †Genes also identified by hybridization of DP2 to the arranged cDNAs.

sion difference between unstimulated and cytokine-stimulated HBEC mRNAs (Table 2). All of the genes that showed differential expression by Northern blotting had an increased abundance in tester compared with driver cDNA when analyzed by virtual Northern blotting except for EBI3 and MMP3, which were not detected or tested, respectively, on virtual Northern blots (Table 2). We were not able to detect significant differential expression for five transcripts that had an increased abundance in tester compared with driver cDNA when analyzed by Northern blotting. This sim-

ply may be the consequence of transcript levels not having been increased sufficiently for visible detection (Table 2). The data for the transcripts that were detected by Northern blotting indicated that ~70% of the genes identified by the cDNA-RDA experiments were indeed differentially expressed. To confirm differential expression for several genes in which expression was only weakly detected by Northern blotting, we performed semiquantitative RT-PCR relative to GAPDH transcript levels. These data indicated that the genes represented by the clones for EXER107, EBI3, and

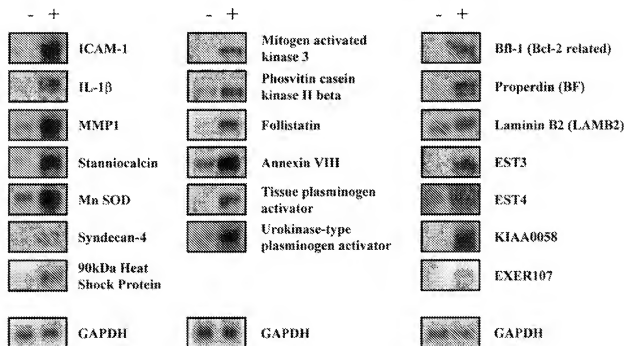


Fig. 1. Virtual Northern analysis of individual clones. Inserts from individual clones isolated from difference product 2 (DP2) were used as hybridization probes on virtual Northern blots containing cDNA from unstimulated (-) and tumor necrosis factor (TNF)- α and interleukin (IL)-1 β -stimulated (+) human bronchial epithelial cells (HBECs). Five hundred nanograms of tester and driver representations were loaded in each lane. Loading of Northern blots was corrected for by hybridizing a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to representative blots. ICAM-1, intercellular adhesion molecule-1; MMP1, matrix metalloproteinase 1; Mn SOD, manganese superoxide dismutase; EST3 and EST4, expressed sequence tags 3 and 4, respectively. See text for discussion.

EXER102 were expressed more abundantly in cytokine-stimulated HBECs (Fig. 3).

Several reports (17, 24, 46) have used hybridization to cDNA representations as an indication for differen-

tial expression; however, our data clearly indicate the need to perform Northern analysis or quantitative RT-PCR for confirmation of differential expression. Of 14 genes that clearly showed differential hybridization

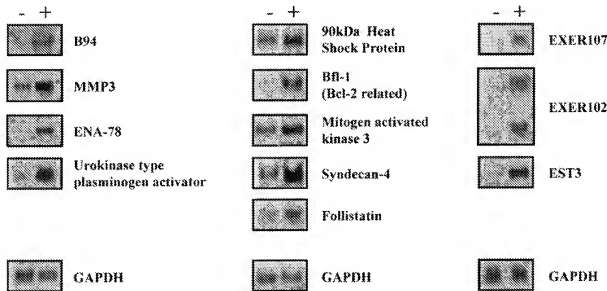
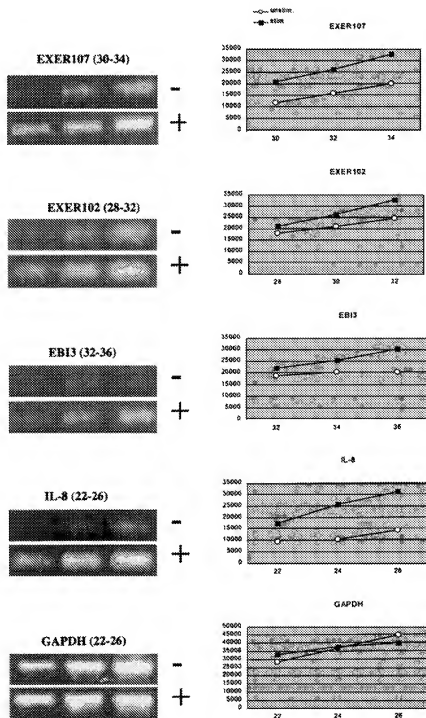


Fig. 2. Northern analysis of individual clones. Inserts from individual clones derived from DP2 were used as hybridization probes on Northern blots containing 10 μ g of total RNA from unstimulated and TNF α - and IL-1 β -stimulated HBECs. Loading of virtual Northern blots was corrected for by hybridizing a GAPDH probe to representative blots. ENA-78, epithelial cell-derived neutrophil-activating protein-78. See text for discussion.

Fig. 3. Semiquantitative RT-PCR was performed relative to GAPDH amplification. *Left*: 100 ng of single-strand cDNA from unstimulated (unstim) and TNF- α - and IL-1 β -stimulated (stim) HBEs were used as templates in both reactions. Seven microliters of amplification product were analyzed on a 1.5% agarose gel. Nos. in parentheses, cycle no. *Right*: individual amplification products were analyzed for integrated pixel intensity (y-axis) and plotted against cycle number (x-axis) with ImageQuant 5.0 software. As a control for HBEc cytokine stimulation, an IL-8 amplification was performed. EB13, Epstein-Barr virus-induced gene 3. See text for discussion.



by virtual Northern analysis, only 9 showed distinct visible differential expression, whereas 5 genes did not when tested on Northern blots.

Tissue distribution of *EST3*, *EXER102*, and *EXER107* expression. Novel genes represented by the clones for *EST3*, *EXER102*, and *EXER107* were further analyzed with multiple tissue Northern blots and RNA master blots. The probe for *EXER102* detected transcripts of ~3 and 6 kb in the majority of mRNAs from adult and fetal tissues (Fig. 4). The

most abundant expression was found in adult and fetal kidneys and adult lung. Analysis of the expression of *EXER102* with the RNA Master blot indicated that the comparatively highest levels of expression were in the trachea, lung, small intestine, kidney, pituitary gland, stomach, thyroid, and fetal lung (results not shown). The probe for *EXER107* detected a single transcript of ~3.5 kb expressed at varying degrees in all adult and fetal tissues; however, a significantly higher abundance of the message was found in adult and fetal

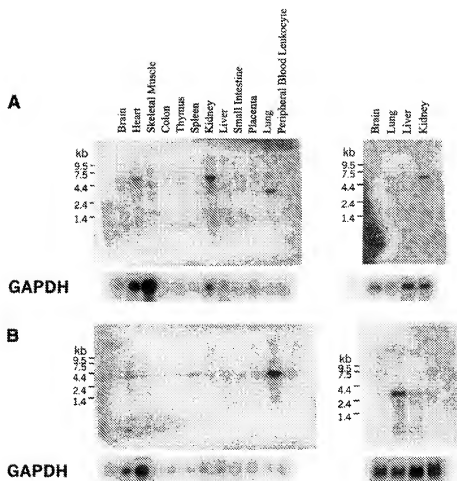


Fig. 4. Northern blot analysis for EXER102 and EXER107. Inserts from representational difference analysis (RDA) clones were hybridized to multiple tissue Northern blots. To control for differences in loading between lanes, the same filters were hybridized with a GAPDH control. mRNA from indicated tissues was loaded in each lane.

lungs (Fig. 4). Analysis of the expression of EXER107 with the RNA Master blot indicated that the comparatively highest levels of expression were found in the trachea, adult lung, kidney, stomach, and thyroid (results not shown). Hybridization of the EST3 probe to multiple tissue Northern blots did not result in the detection of a transcript in any tissue tested; however, hybridization to a RNA Master blot indicated that the gene represented by EST3 was expressed most abundantly in the pancreas, mammary gland, thyroid, bladder, and trachea (results not shown).

DISCUSSION

We performed cDNA-RDA to identify and isolate genes in primary HBECs in which expression is induced by the inflammatory cytokines TNF- α and IL-1 β . We used a mixture of these two cytokines in our experiments because their coexpression and their synergistic effects are important for disease pathology in inflammatory conditions. As expected, we found an induced expression of a number of known inflammation-related genes, including IL-1 β and TNF- α themselves; several genes involved in inflammatory cell trafficking, such as IL-8, regulated on activation normal T cell expressed and secreted (RANTES), and epithelial cell-derived neutrophil-

activating protein-78 (27, 34, 43); and genes involved in the activation of inflammatory cells, such as granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor, that confirms the validity of our approach. In addition, the stimulated airway epithelial cells showed induced expression of a number of protective genes including those for antiproteases, such as the tissue inhibitors of metalloproteinases (5), and antioxidants, such as manganese superoxide dismutase (47). TNF- α is also known for its ability to induce apoptosis in a variety of cell types through its activation of its cognate receptor subtype I (18). One mechanism by which the airway epithelial cells may protect themselves against TNF- α -induced apoptosis is by expressing Bfl-1/A1, a protein that is able to block cellular apoptosis. This prosurvival Bel-2 homolog is a direct transcriptional target of NF- κ B, hence providing evidence supporting the protective role of NF- κ B in the immune system (51). Another gene involved in apoptosis regulation that we identified as being upregulated by TNF- α and IL-1 β is the gene for the 90-kDa heat shock protein (HSP90). HSP90 functions as a molecular chaperone that aids in the folding of nuclear hormone receptors and protein kinases. A study (19) has shown that an excess of HSP90 is associated with increased apoptosis

and, conversely, that reduced HSP90 levels correlate with protection against apoptosis in TNF- α - and cycloheximide-treated cells (19). It therefore seems possible that in bronchial epithelial cells exposed to inflammatory conditions, a similar fine balance between survival and apoptosis may exist. In vivo, when this balance is shifted in favor of apoptosis, epithelial cell shedding may occur as seen in the asthmatic condition (25).

We also identified two kinases involved in intracellular signaling as having upregulated transcription on cytokine stimulation. Previous work (48) has shown that mitogen-activated protein (MAP) kinase 3 (MKK3) is a critical component of a TNF- α -stimulated signaling pathway that causes increased expression of inflammatory cytokines. Targeted disruption of the MKK3 gene resulted in a selective defect in the response of fibroblasts to TNF- α , including reduced p38 MAP kinase activation and cytokine expression (48). However, its upregulation in inflamed airway epithelial cells has not been previously reported. Phosvitin/casein kinase II (CKII) was also shown to be present in DP2 (Table 2). This kinase has previously been shown to immunoprecipitate as part of the NF- κ B cytoplasmic complex, and phosphorylation of NF- κ B by CKII may control translocation of the activated complex or modulate its ability to bind DNA (4, 44).

The cell surface proteoglycan syndecan-4 was identified as being upregulated in HBECs by cytokine stimulation. This molecule has the ability to regulate cell adhesion via its glycosaminoglycan chains and the discrete domains of its core protein; it is also able to bind and activate protein kinase C- α (21). Previous work in vitro (50) has shown that TNF- α is able to induce endothelial cell syndecan-4 expression by both increasing gene expression in a NF- κ B-dependent manner and prolonging mRNA half-life. In vivo studies have shown that levels are also increased after various forms of tissue injury including skin wounds, vascular wall injury, or myocardial infarction (50). Both RT-PCR and Northern analysis (results not shown) indicated that the transcript levels for another cell surface molecule, the Epstein-Barr virus (EBV)-induced gene 3 (EBI3), were also increased in cytokine-stimulated HBECs. EBI3 encodes a 34-kDa glycoprotein, a member of the hematopoietin receptor family related to both the p40 subunit of IL-12 and the diliary neurotrophic factor receptor. The protein lacks a membrane-anchoring motif and is secreted but is also present on the plasma membrane of EBV-transformed B lymphocytes and transfected cells (12). Latent membrane protein 1 of the EBV has been shown to induce the expression of EBI3 along with ICAM-1, lymphocyte function-associated antigen-3, and CD40 in EBV-negative Burkitt lymphoma cells and in epithelial cells via NF- κ B activation. (13). Consistently, our data indicate that this gene can also be induced in HBECs by the two cytokines TNF- α and IL-1 β , suggesting a possible role for this molecule in respiratory inflammation.

Follistatin is a secreted protein that is able to bind and neutralize the actions of many members of the transforming growth factor- β family of proteins. Fol-

listatin is known to bind activin, a member of this family, and both are upregulated by mediators of inflammation (36). Our data are, however, the first to suggest that follistatin plays a possible role in airway inflammation. The urokinase-type plasminogen activator (uPA) and the uPA receptor (uPAR) are thought to be critical in the cell invasion processes. The balance between uPAR-bound uPA and its inhibitors modulates a pericellular proteolytic activity able to give "stop-and-go" signals to invading cells, possibly in concert with the matrix metalloproteinase system. Other lines of evidence suggest that the uPA-uPAR interaction elicits chemotaxis, chemoinvasion, and cell multiplication, with uPA also being implicated in the inflammatory disease rheumatoid arthritis (11). Our data now suggest that the uPA may also have a role in lung inflammation. The tissue-type plasminogen activator (tPA) gene was also identified as being regulated by TNF- α and IL-1 β . tPA has been shown to have anti-inflammatory properties, and a study (42) suggested that tPA may suppress neutrophil activation in vivo but does not reduce lung neutrophil infiltration. Indeed, treatment with tPA has been shown to significantly improve the mortality rates of patients with acute myocardial infarction, and the potential for tPA treatment in other inflammatory diseases such as acute respiratory distress syndrome and rheumatoid arthritis, in which neutrophil-mediated injury is likely, has been suggested (41). Our findings add further support for its use as a possible treatment for certain lung inflammatory diseases.

Several cDNA fragments that contained the R-Bgl-24 adapter sequence were identified from DP2. These cDNA fragments would effectively behave as tester-unique fragments in the subtractive hybridization steps. The most likely explanation for the presence of these clones in DP2 may be due to inefficient digestion by the *DpnII* restriction enzyme. Several cDNA fragments that represent genes generally classified as "housekeeping" were also identified from DP2 (see Table 2). These may also be artifacts of the experimental process not being successfully eliminated during the subtraction procedure. However, identification of cDNAs for housekeeping genes among subtraction products has previously been reported by several groups including our own (10, 17, 20); however, further rounds of subtraction may result in the elimination of these genes. Alternatively, it is also possible that some of these genes are actually induced by cytokine treatment and may indicate a change in the metabolic activity of cytokine-stimulated HBECs (6).

Our studies demonstrate that hybridization of DP2 to arrayed cDNAs allowed rapid identification of the genes represented and provided information regarding the success of the subtraction experiment. This approach identified several genes including those for E-selectin, Zfp-36, monocyte chemoattractant protein-1, vascular endothelial growth factor, and RANTES (Table 1), all having been previously reported to be regulated by TNF- α and/or IL-1 β (22, 35, 37, 38); however, these genes were not identified when we sequenced our

1,152-clone library. Differing cloning efficiencies of individually amplified fragments may possibly explain the reason for this discrepancy. Although hybridization to gene arrays can be used to rapidly identify some of the genes represented in a complex (DP2), it is unlikely to be able to identify all of the genes represented until all the genes within the genome have been identified and subsequently arrayed. Our data therefore support the notion that hybridization of DP2 to gene arrays combined with cloning and sequencing may provide a more comprehensive analysis of the subtraction product.

A full-length uncharacterized gene, KIAA0058, was also identified as being represented in DP2. Northern blot analysis has previously shown this gene to be expressed in a variety of tissues (31). The mouse homologue, designated prtb (proline codon-rich transcript, brain expressed) has also been recently identified, and knockout mice have been generated. These mice were viable and fertile and did not display any obvious abnormalities (49). Our data suggest that KIAA0058 may play a role in lung inflammation (Fig. 1); however, a more detailed analysis of the lung function and bronchial hyperresponsiveness of the prtb knockout mice may provide further insight into its role.

Northern analysis of the gene fragments representing genes of unknown function, EXER107 and EXER102, indicated that in adult tissue they are predominantly expressed in the lung (Fig. 3), with EXER107 also appearing important for fetal lung development (Fig. 3). Although we detected a hybridization signal in total RNA derived from HBECS for EST3 (Fig. 2), unexpectedly, we were not able to detect any signal in adult or fetal tissues. This result may be explicable due to EST3 having a restricted pattern of expression unique to HBECS, whereas EXER102 and EXER107 may be expressed in other cells of the airways and hence give detectable hybridization intensities in complex tissues. Sequences of EST3, EXER102, and EXER107 showed no similarity to any genes of known function in the public databases, and, therefore, we are not able to speculate further on their potential roles. Further work needs to involve isolation of full-length sequences for these clones, and this may provide further insight into their action. A thorough and quantitative mapping of the gene expression pattern of our panel of genes in various (patho)physiological situations will give indispensable information concerning inflammatory conditions of the lung. This information, in turn, may help us devise new rational and gene therapeutic approaches for the treatment of various respiratory diseases.

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